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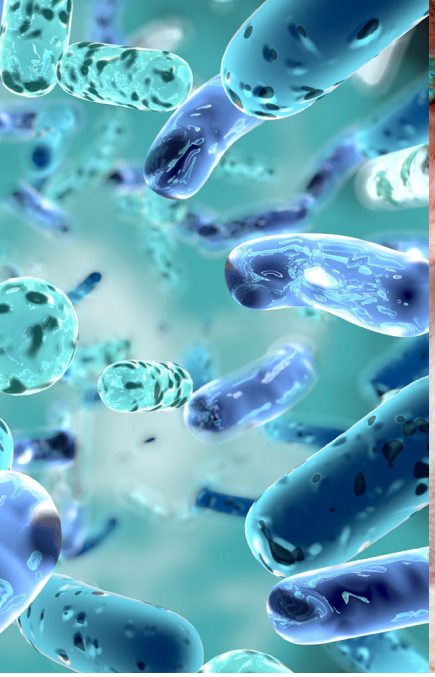
White, John Kerr

Publication date:
2020

Document Version
Publisher's PDF, also known as Version of record



MICROORGANISMS IN OCCUPATIONAL SETTINGS

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BY
JOHN KERR WHITE

DISSERTATION SUBMITTED 2020

AALBORG UNIVERSITY
DENMARK

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Dissertation submitted August 2020

Dissertation submitted: 31st of August, 2020

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PhD Series: Faculty of Engineering and Science, Aalborg University

Department: Department of Chemistry and Bioscience

ISSN (online): 2446-1636
ISBN (online): 978-87-7210-697-7

Published by:
Aalborg University Press
Kroghstræde 3
DK – 9220 Aalborg Ø
Phone: +45 99407140
aauf@forlag.aau.dk
forlag.aau.dk

Printed in Denmark by Rosendahls, 2020

ENGLISH ABSTRACT

Workers on pig farms are well known to be exposed to high levels of airborne bacteria, fungi, and endotoxin which can affect their health. Previous research has focused on specific measurements, such as concentration of airborne dust, endotoxin, or exposure to specific species of bacteria or fungi. By focusing on specific species, there runs the risk that medically relevant species or antimycobial-resistant fungi will be ignored. Therefore, the current methods used to characterise the workers' occupational exposure may not be sufficient to adequately assess the risk associated with workers' occupational exposure to airborne microbes.

The objectives of this Ph.D. thesis were therefore to assess additional risk assessment parameters which could be used in the future development of a more well-rounded and comprehensive occupational risk assessment strategy. A combination of air and dust samples from pig farms and laboratory-scale experiments were used in this Ph.D.'s four studies for characterising diversity of microorganisms in pig farm dust, the biofilm forming ability of aerosolised bacteria, viability of airborne microbes, and presence of antimycobial resistant fungi.

In the first study, the microbial communities, bacterial and fungal, from five pig farms were characterised using molecular and culture-based techniques. The results from this study showed that the five pig farms had highly dissimilar bacterial community structures, indicating that hygienic strategies for reducing workers' exposure to certain bacteria might not be applicable to other pig farms. In contrast, the fungal communities showed greater similarity, showing that there were more shared fungal species between pig farms, thereby indicating that pig farm workers' from different farms might experience similar exposures.

The bacterial pathogen *Staphylococcus aureus* is commonly encountered in the pig farm environment in airborne dust. During the second study, the potential of aerosols containing *Staphylococcus aureus* generated in an aerosol chamber, to form a biofilm once collected was assessed. Biofilms have not been a focus for occupational hygiene studies. However, the results from this Ph.D. supports studying the potential for bioaerosols to form biofilm, as the aerosolised *S. aureus*, when exposed to airborne dust, formed more biofilm than bacterial aerosols which were not exposed to airborne dust. It is already known that work in farms can lead to the development of chronic airway diseases, such as asthma and chronic obstructive pulmonary disease (COPD). In addition, biofilm related infections are more difficult to treat with antibiotics.

Assessments of the microbial burden that workers face are often based on culturing of the bacteria, and fungi present in airborne or settled dust. However, with the advent of molecular based technologies, such as PCR, it became possible to specifically target low abundant, but clinically and occupationally relevant species of bacteria and

fungi. Unfortunately, these techniques are prone to bias as they target both living and dead cells, which might skew the final signal and lead to erroneous conclusions. For these reasons, during the second and third studies, the viability of airborne bacteria *in vitro* and fungal aerosols *in natura* was assessed to understand how aerosolisation and time affect the survival of different microbial species. During the second study, the results suggested that aerosolisation might induce the viable, but not culturable (VBNC) state in *Staphylococcus aureus*. Furthermore, during the third study, it was found that a large proportion of airborne fungi in the studied pig farm were non-viable, and that these genera were commonly associated with the pigs' gastrointestinal tract. In contrast, several of the viable fungal genera were known to contain pathogenic species, which can cause severe lung infections in immunocompromised hosts, and could deposit in the upper airways.

Although it is well known that resistance to antimycobials has been rising on a global scale, it has not been studied whether work in pig farm stables can lead to exposure to antimycobial-resistant fungi. Air and environmental samples were collected from a pig farm over a period of three sampling days. By applying a known strategy for testing antimycobial-resistance in clinical isolates, it was found that several isolates of *Aspergillus spp.* were resistant to medical antimycobials. This is worrying, as it means that pig farm workers are at an increased risk of developing an occupationally derived antimycobial-resistant fungal infection.

Overall, this Ph.D. provides new potential test strategies designed towards the development on an appropriate risk evaluation for pig farm workers' potential exposure to airborne microorganisms when working in pig stables. The results from this Ph.D. show that there are parameters, including studying microbial viability, potential to form biofilm, and presence of antimycobial-resistant fungi, which can be relevant to consider when making a risk evaluation.

DANSK RESUMÉ

Det er velkendt at folk der arbejder i svinestalde bliver udsat for høje koncentrationer af luftbårne bakterier, svampe og endotoksin som kan påvirke deres helbred. Forskning har været fokuseret på specifikke målinger, så som koncentration af luftbårent støv, endotoksiner eller medarbejdernes udsættelse for bestemte bakterier eller svampe. Ved at kigge efter specifikke arter overser man måske arter der kan være af helbredsmæssig betydning eller isolater der er resistente over for svampecider. Derfor er de nuværende metoder, der bruges til at karakterisere de ansattes arbejdsrelaterede eksponering, er muligvis ikke tilstrækkelige til at lave en ordentlig risikovurdering vedrørende svinebedriftsarbejderes udsættelse for luftbårne mikroorganismer.

Formålet med denne ph.d. er derfor at undersøge andre parametre der kan blive brugt til fremtidig udvikling af en mere tilstrækkelig og omfattende strategi indenfor arbejdsrisikovurdering. En kombination af luft- og støvprøver og laboratorie-eksperimenter blev gennemført i denne ph.d.'s fire studier til at karakterisere diversiteten af mikroorganismer i støv fra svinestalde, aerosolisede bakteriers biofilmdannelsesevne, luftbårne mikroorganismers levedygtighed og tilstedeværelsen af svampecid-resistente svampe.

I det første studie blev de mikrobielle samfund fra fem svinegårde karakteriseret med molekylær- og dyrkningsbaserede metoder. Resultaterne fra studiet viste, at de fem gårde havde hver deres egne unikke bakterielle samfund, hvilket indikerer, at hygiejnestrategier til at reducere medarbejdernes udsættelse for bestemte bakterier ikke nødvendigvis kan overføres fra den ene gård til den anden. Derimod var der høj overensstemmelse mellem de observerede svampe-samfund på de fem gårde, hvilket indikerer, at ansatte i forskellige svinegårde udsættes for en svampeeksponering der ligner hinanden.

Det bakterielle patogen *Staphylococcus aureus* forekommer hyppigt i svinestalde i luftbårent støv. Det andet studie undersøgte, om aerosoler med *S. aureus,* genereret i et aerosolkammer, efter opsamling kunne danne biofilm. Biofilme har ikke været en fokus i arbejdshygiejniske studier. Men resultater fra dette studie peger frem mod nye studier om emnet idet *S. aureus* aerosoliseret sammen med støv, dannede mere biofilm end bakterien aerosoliseret alene. Det er allerede kendt, at arbejde i svinestalde kan føre til udvikling af kroniske luftvejsproblemer, såsom astma og kronisk obstruktiv luftvejssygedom (KOL). Desuden er en infektion med biofilmdannelse sværere at bekæmpe med antibiotika.

Eksponering for svampe og bakterier i svinestald er ofte målt ved dyrkning af de bakterier og svampe, der var tilstede i luftbårent eller sedimenteret støv. Men med udviklingen af molekylærbaserede metoder, som PCR, blev det muligt til at fokusere

på arter med lav forekomst, som er kliniske og arbejdsrelaterede. Desværre er disse metoder tilbøjelige til fejltolkninger, da de fokuserer på både levende og døde celler, hvilket kan føre til fejlagtige handlingsplaner. Af den grund blev levedygtigheden af luftbårne bakterier *in vitro* og svampeaerosolser *in natura* i det andet og tredje studie analyseret for at fremme forståelsen af, hvordan aerosolisering og tid påvirker forskellige mikrobielle arters overlevelse. Resultaterne fra det andet studie antydede, at aerosolisering inducerede det levende, men ikke dyrkbar stadie af *S. aureus*. I det tredje studie blev det desuden vist, at en stor del af de luftbårne svampe fra den undersøgte svinegård ikke var levedygtige, og at de indsamlede svampeslægter var associeret med svinenes tarmflora. I kontrast til disse observationer, blev det påvist, at mange af de levedygtige svampe inkluderede patogener, hvilket kan føre til alvorlige lungesygedomme i immunkomprimerede værter, og at disse kunne deponeres i menneskers øvre luftveje.

Selvom at det er velkendt, at resistens mod svampecider stiger på en globalt, er det ikke undersøgt om arbejde i svinestalde kan føre til udsættelse for svmape, der er resistente mod medicinske svampecider. Derfor, fokuserede den sidste del af denne ph.d. på at undersøge om der er svampecid-resistente svampe i en svinestald. Luft- og materialeprøver blev indsamlet igennem tre måledage. Ved at anvende en kendt strategi til at teste svampecid-resistens i kliniske isolater, blev det vist, at adskillige isolater af *Aspergillus spp.* var resistente over for medicinske svampecider. Dette er foruroligende, da det betyder, at der ved arbejde i svinestald er en øget risiko for at udvikle en erhvervsmæssigt afledt svampecid-resistent svampeinfektion.

Samlet set, fremstiller denne ph.d. nye potentielle teststrategier designet til udvikling af en passende risikovurdering af den potentielle eksponering for luftbårne mikroorganismer ved arbejde i svinestalde. Resultaterne fra denne ph.d. viser, at der er parametere, som undersøger mikroorganismers levedygtighed, biofilmdannelseevne, og tilstedeværelse af svampecid-resistente svampe, der kan være relevante at tage med i en risikovurdering

PREFACE

This dissertation is submitted in partial fulfilment for the requirements for obtaining the degree Doctor of Philosophy (Ph.D.). The thesis describes the outcomes of the study Microorganisms in Occupational Settings: Working towards an evaluation of risks and exposure during work with animals, carried out from August 1st 2017 to the 31st of August 2020 at Aalborg University (AAU), at the Department of Chemistry and Bioscience in addition to the National Research Centre for the Working Environment (NRCWE). The results presented here were obtained as part of the Danish Working Authority. The thesis consists of an extended introduction that summarises the background and literature relevant to the Ph.D. thesis, supported by four scientific papers, included as appendices.

First and foremost, I would like to give my sincerest thanks to both of my supervisors, Professor MSO Jeppe Lund Nielsen and Senior Researcher Anne Mette Madsen, for giving me the opportunity to work on this project. The discussions we have had, academic or otherwise, have helped inspire me and improve my knowledge and understanding of aero-microbiology and occupational hygiene.

Secondly, I would like to thank my colleagues at AAU and NRCWE, both past and present, for all the help and support I have received throughout the years. Special thanks to Nadieh from AAU for helping me whenever I had to sequence my samples. I would like to thank Rui and Pil from the NRCWE for helping me whenever I was about to murder my computer, and Margit, for always helping me in the lab including that one time I forgot to put the hose from the agar-clave in the sink and we ended up having to wash the floor. My bad. I would like to thank Trine Larsen from the NRCWE, who helped me during this Ph.D., and produced diagrams and schematic representations.

I would like to extend my thanks to my friends, who listened to me whenever I was complaining about my fungal cultures and keeping me sane during the last few years, which has been invaluable for me completing this Ph.D.

I would like to thank my mom, dad, and sister for helping me chase my dreams in academia. Without your support, I would have never made it this far.

Finally, I would like to thank you, the reader, for taking the time to read this thesis.

I hereby declare that this is my original work.

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OBJECTIVES OF THE PH.D. PROJECT

The overall objectives of this Ph.D. project were to assess parameters which could be used for studying pig farm workers' exposure to microorganisms in pig farms. This was done by using biofilm formation assays, next-generation molecular techniques for assessing microbial viability, and antimycobial resistance testing. These parameters were taken into consideration, all in combination with multivariate statistical analyses, to study the potential risk workers may face when working with pigs, with focus on airborne bacterial and fungal exposure. Investigations were performed on field samples from several farms and with laboratory-scale aerosol chamber experiments.

The aims of the individual studies included in this Ph.D. thesis were the following:

Paper 1	**Microbial species and biodiversity in settling dust within and between pig farms**
This study aimed to elucidate the microbial communities (bacterial and fungal) present in the settling dust from five different pig farms in Denmark, in regards to their diversity and species composition, as no broad information regarding the microbes, pathogens and non-pathogens, present in pig farm dust is available. Due to this lack in knowledge, occupational hygienists do not know whether strategies used to reduce workers' exposure to certain microbes on one farm will be applicable for other farms. In addition, because the microbial diversity in pig farm dust is not well characterised, patients with an occupationally derived infection may not receive appropriate treatment in due time as their medical provider may not be sure what they were exposed to.	
Paper 2	**Impact of dust on airborne *Staphylococcus aureus'* viability, culturability, inflammogenicity, and biofilm forming capacity**
Staphylococcus aureus is a common airborne pathogen found in Danish pig farms, and workers are known to be at an increased risk of being colonised by this species. The goal for this study was to assess the culturability, viability, biofilm forming capacity, and inflammatory potential of *S. aureus* when aerosolised with or without the presence of sterile airborne pig farm dust. The results from this study suggested, that when *S. aureus* is aerosolised, it might enter the viable, but not culturable (VBNC) state, and therefore would not be quantifiable when using culturing dependent methods. Additionally, the presence of airborne dust increased the culturability, amount of biofilm formed, and the inflammogenicity of aerosolised *S. aureus.*	

Paper 3	**Potential respiratory deposition and species composition of airborne culturable, viable, and non-viable fungi during occupancy in a pig farm**
Although it is known that pig farmers are exposed to elevated concentrations of airborne fungi, there is a lack of knowledge on where in the human airways different airborne fungi may deposit. This presents a significant issue, as certain fungal species can cause different diseases based on where in the airways they may deposit. In this study, the species of airborne fungi from a pig farm were identified in regards to their potential deposition within the airways and analysed for which fungi were viable at the time of samples. The results showed, that workers were at risk of being exposed to varying concentrations of pathogenic and allergenic species of fungi, which exhibited potential to deposit in all regions of the airways. In addition, many of the fungi that workers were potentially exposed to were non-viable and these fungi were typically species known to be associated with the pig gastrointestinal tract.	
Paper 4	**Paradoxical and classical antifungal resistance in environmental isolates of *Aspergillus***
Previous research conducted during this Ph.D. has shown that pig farm workers are potentially exposed to airborne pathogenic fungi, and other work has shown that antimycobial resistance is spreading throughout Europe. In this study isolates from the genus *Aspergillus* were tested for their resistance to medical antimycobial drugs used in patients to see whether pig farm workers are exposed to antimycobial resistant fungi, which can cause serious disease.	

Knowledge and results gathered in this Ph.D. are proposed to be implemented in the future development of risk assessment analyses for workers potentially exposed to microorganisms when in close contact with animals.

LIST OF SUPPORTING PAPERS

This thesis is based on the following four scientific papers:

Paper 1:

Microbial species and biodiversity in settling dust within and between pig farms.
John Kerr White, Jeppe Lund Nielsen, Anne Mette Madsen (2019).
Published in *Environmental Research*

Paper 2:

Impact of dust on airborne *Staphylococcus aureus'* viability, culturability, inflammogenicity, and biofilm forming capacity
John Kerr White, Jeppe Lund Nielsen, Cecilie Møller Larsen, Anne Mette Madsen
Accepted for Publication in *International Journal of Hygiene and Environmental Health*

Paper 3:

Potential respiratory deposition and species composition of airborne culturable, viable, and non-viable fungi during occupancy in a pig farm
John Kerr White, Jeppe Lund Nielsen, Anne Mette Madsen (2020).
Published in *Atmosphere*

Paper 4:

Paradoxical and classical antifungal resistance in environmental isolates of *Aspergillus*
John Kerr White, Jeppe Lund Nielsen, Jan Struckmann Poulsen, Anne Mette Madsen
Manuscript in preparation

LIST OF OTHER SCIENTIFIC WORK

The following publications have been contributed to during the Ph.D., but are not aligned with the scope of the thesis.

Exposure characteristics of airborne bacteria during a haze pollution event at Qinling Mountain, China
Rui Lu, Chunlun Fan, Pengxia Lu, Yuzhen Qi, Feifei Mu, Zhensheng Xie, **John Kerr White**, Anne Mette Madsen, Yanpeng Li (2019).
Published in *Human and Ecological Risk Assessment (2019)*

Evaluation of methods for sampling of *Staphylococcus aureus* and other *Staphylococcus* species from indoor surfaces.
Anne Mette Madsen, Hoang U. T. Phan, Mathias Laursen, **John K. White**, Katrine Uhrbrand (2020).
Published in *Annals of Work Exposures and Health (2020)*

A cohort study of cucumber greenhouse workers' exposure to microorganisms as measured using NGS and MALDI-TOF MS and biomarkers of systemic inflammation
Anne Mette Madsen, **John Kerr White,** Amal Markouch, Sarah Khadim, Nadieh de Jonge, Trine Thilsing, Vinni Mona Hansen, Jesper Bælum, Jeppe Lund Nielsen, Ulla Vogel, Kira Tendal.
Submitted to *Environmental Research*

CHAPTER 1. INTRODUCTION

The air surrounding us is a complex mixture of chemicals in gaseous state and suspended solids which are both organic and inorganic in nature (Glueckauf, 1951). These compounds include, but are not limited to, ammonia, endotoxin, dust, volatile organic compounds (VOCs), and airborne microorganisms, such as bacteria and fungi (Chmielowiec-Korzeniowska et al., 2018; Heederik et al., 1991). In most environments, these particles are not present in high concentrations, but in certain environments, such as pig farms and other occupational settings, the concentrations of these particles are higher than normal and can begin to cause detrimental health effects in workers. For pig farm workers, exposure to high concentrations of microorganisms is well known to be linked with the development of occupational disease(s) such as organic dust toxic syndrome (ODTS) (Donham et al., 1995), hypersensitivity pneumonitis (Vogelzang et al., 2000), asthma (Randell and Boucher, 2006), and potential infection or colonisation by pathogenic microorganisms (Angen et al., 2017).

However, for most farmers and other farm workers, the likelihood of developing a serious infection from exposure to airborne microorganisms at work is relatively low, as healthy hosts are able to clear pathogenic or allergenic bacteria and fungi from their airways (Park and Mehrad, 2009; Randell and Boucher, 2006). Despite this, a series of different studies has shown, that although the workers may not develop infections, they may still be carriers of pathogens such as livestock associated methicillin resistant *Staphylococcus aureus* (LA-MRSA). In one study, volunteers who were MRSA negative prior to entering an LA-MRSA positive pig farm subsequently tested positive for LA-MRSA upon leaving (Angen et al., 2017). Workers who work with LA-MRSA positive herds have been found to be more likely to test positive for MRSA than workers who are not in contact with swine (Wardyn et al., 2015). Thus pig farming presents as an occupational risk, as workers have an increased risk of developing an LA-MRSA infection or becoming colonised by it.

Despite this wealth of knowledge on workers' exposure to specific pathogens such as LA-MRSA, there exists a gap in the current scientific literature regarding the proper development of a risk exposure assessment for pig farm workers. Therefore, this warrants further study.

1.1. PIG FARM WORKERS' POTENTIAL EXPOSURE

The question remains; "why study pig farm workers' exposure"? In relation to the size of its population, Denmark is one of the world's leading producers of pigs for slaughter and sale. In 2019, 12.6 million pigs were raised (Danmarks Statistik, 2020), as seen in Figure 1. Due to the high levels of production, pig farming represents an

important economic resource, as it supports approximately 33,000 jobs and contributes approximately 18-19 billion DKK per year to the Danish economy (Landbrug og Fødevarer, 2017).

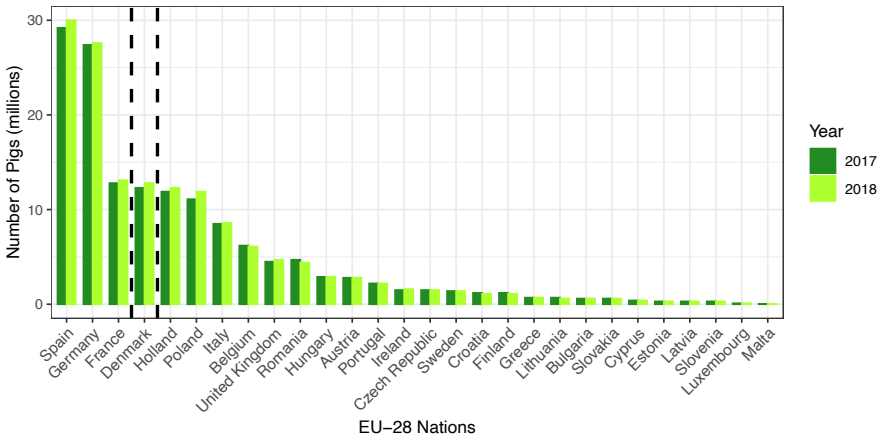

Figure 1 Number of pigs produced by each member of the European Union (EU) in 2017 (dark green) and 2018 (light green). Denmark is highlighted between the two dashed-lines. Data adapted from Danmarks Statistik

Despite pig farming representing an important economic resource, detailed research on pig farmers' exposure to airborne microorganisms has not been a priority, likely due to the lack of infections amongst swine producing farmers. Although the farmers may not experience acute infection, they are still subjected to high exposure levels of potentially pathogenic bacteria and fungi, and the farm workers can become colonised by these species. Thus, if the workers' immune system becomes comprised then there is an increased chance for a serious infection (Corbella et al., 1997; Davis et al., 2004). In addition, it is well known that farmers' exposure to airborne microorganisms is linked to the development of chronic obstructive pulmonary disease (COPD), asthma, and reduced lung function (Heederik et al., 1991; Monsó et al., 2004; Zhiping et al., 1996).

Of the studies which have been performed on pig farm workers' exposure, these do not necessarily take into consideration a workers' total exposure, as several studies prioritise certain exposures, such as specific bacterial species, endotoxin, dust, and chemical exposure (Angen et al., 2017; Sowiak et al., 2011; Vogelzang et al., 1998). Therefore, there is a need for studying pig farm workers' exposure to the different species of bacteria and fungi that they are exposed to.

1.2. FUNGAL AND BACTERIAL COMMUNITIES IN PIG FARMS

As previously mentioned, pig farmers are exposed to elevated concentrations of a variety of different chemical and biological agents. However, the understanding of the potential risks associated with microorganisms is not well established, and therefore warrants the need for further occupational hygiene studies. The aim of occupational hygiene studies is to prevent, or develop tools intended to prevent, workers from becoming ill due to their work conditions, via the recognition and evaluation of negative work factors or agents (Oppliger and Seixas, 2017). In the case of pig farm workers, one of these occupational agents involves exposure to elevated concentrations of microorganisms.

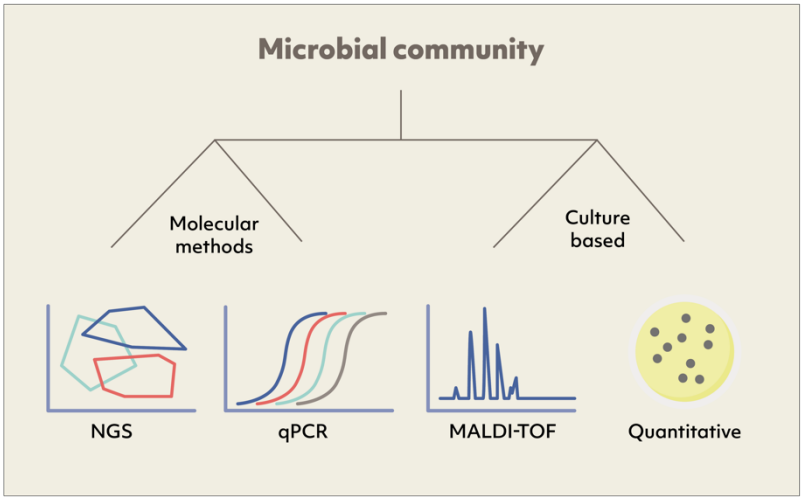

Figure 2 A simplified and brief overview of the molecular- and culture-based methods used for characterising the airborne microbes (bacterial and fungal) that pig farm workers are exposed to. Figure provided by Trine Larsen.

In brief, in many occupational hygiene studies, workers' exposure to bacteria and fungi are measured by sampling sedimented or airborne dust and analysing the contents using culture-based or molecular-based methods. An illustrative overview of the different ways of characterising the potential microbial exposure pig farm workers face is shown in Figure 2. Of the studies assessing the microbial burden pig-farm workers face, these have relied heavily on culturing and microscopy to identify airborne or sedimented bacteria and fungi (e.g., Kristiansen et al., 2012; Sowiak et al., 2011; Viegas et al., 2013). However, the level of taxonomic resolution that these studies were able to obtain was generally limited due to difficulty in differentiating between closely related or cryptic species, and in the limited percentage of microbes which are culturable in laboratory conditions.

The information gained from these earlier studies is highly valuable, as it has provided a solid foundation for researchers and workers to begin to consider methods for reducing pig farm workers' exposure to airborne bacteria and fungi. Yet, those earlier studies were unable to gain data regarding the specific species and genera of bacteria and fungi, workers were exposed to. This presents as a problem, as in order to make a proper risk assessment, knowledge on the species of bacteria and fungi is vital, as not all species of bacteria and fungi are pathogens and some pose a much greater risk than others. For example, the bacterial genus *Staphylococcus* is composed of several species, some of which are human pathogens, but there are also many species of staphylococci which are non-pathogenic for humans (Rosenstein and Götz, 2013). As a real-world example of the applicability of this, a study on the bacterial composition in pig farm dust found high concentrations of staphylococci within the dust when using methods with limited taxonomic resolution (next generation sequencing), but when using a method with a higher taxonomic resolution (in this case MALDI-TOF MS), it was revealed that a large proportion of the bacteria were non-pathogenic species within the genus (**Paper 1**). Therefore, if the study had only relied on the method which obtained lower taxonomic resolution, potentially erroneous conclusions regarding the workers' occupational risk could have been made.

This represents an important development in occupational exposure assessments, as with the development and advances in modern techniques, such as next generation sequencing (NGS) and matrix assisted laser desorption/ionisation time of flight mass spectrometry (MALDI-TOF MS), the speed and practicality of performing large scale microbial ecology studies have become feasible, and allow for high taxonomic resolution, which is invaluable for an appropriate risk evaluation.

The use of MALDI-TOF MS for the identification of bacteria and fungi within complex samples has allowed for labs to take a small step from only quantifying the number of colony forming units (CFUs) to obtaining species level identification. This represents an important step in occupational exposure studies, as earlier studies were unable to quickly obtain high taxonomic resolution data regarding the species of bacteria and fungi that workers were exposed to. MALDI-TOF MS was used for characterising the bacteria and fungi cultured from sedimented dust in **Paper 1** (White et al., 2019), and used for obtaining species level identification from airborne fungi in **Paper 3** (White et al., 2020a). However, relying solely on MALDI-TOF MS for occupational exposure characteristics is still not without limitations; not all species of bacteria and fungi are culturable (the so called "great plate-count anomaly") and under certain culturing conditions, such as under anaerobic/aerobic conditions, not all species of bacteria and fungi will be able to form colony forming units.

In contrast to culture-based methods, such as MALDI-TOF MS, NGS-based studies primarily rely on ribosomal genes and internal transcribed spacer (ITS) regions for the identification of bacteria and fungi, respectively. The 16S rRNA gene is the preferred gene for obtaining taxonomic identification of prokaryotes, as it contains

highly conserved regions (allowing for universal primer design) which border nine hypervariable regions. Sequencing of these different regions allows for discrimination between closely related organisms (Woese, 1987). In contrast, the fungal ITS region has fewer hypervariable domains, and the size and copy number varies wildly between closely related species (Lofgren et al., 2019). Despite this, the ITS region remains widely used as the genetic element used for fungal microbial ecology (Schoch et al., 2012).

Sequencing methodologies and the application of these techniques to occupational settings have rapidly evolved over the last decade, allowing for greatly improved throughput and yield of molecular analyses. For example, the bacteria and fungi present in dust from pig farms has been sequenced, as analysed in **Paper 1**, revealing a large diversity of bacterial and fungal genera (White et al., 2019). In addition, the sequencing of nasal swabs from pig farmers and non-farmers has shown differences in the nasal microbial communities, which has been attributed to differences in occupation (Kraemer et al., 2018).

This is not to say, that sequencing methods are without their own limitations; in particular, the microbial communities observed after sequencing are known to be affected by factors such as DNA extraction choice, choice of DNA primer region, interpersonal bias, and choice of sequence reference database (Albertsen et al., 2015). Furthermore, bacteria possess between 1 and 15 copies of the 16S rRNA gene (Větrovský and Baldrian, 2013), and some fungal species can exhibit varying copies of their chromosomes with multiple, or single copies of their genome present (Todd et al., 2017). To further confound this, not all species of fungi have the same number of rRNA operons in their genome (Lofgren et al., 2019). Due to these differences in target region copy number, species with different target region copy numbers will be over, or under-represented in the final dataset. These confounding variables accumulate and result in datasets, which are at best, only semi-quantitative.

Due to the strengths and weaknesses of relying on a single method for characterising the microbes which workers may be exposed to, other studies have shown that using both MALDI-TOF MS and NGS can support the data obtained from either method (Madsen et al., 2015). This is supported in **Paper 1**, where MALDI-TOF MS and NGS were used to characterise the microbes present within settled dust. Using culturing in combination with MALDI-TOF found that the dust contained a high concentration of skin and dust associated bacteria, including *Aerococcus viridans* and *Staphylococcus equorum*. However, sequencing revealed that the majority of the microbes which were present in the dust were associated with the gastrointestinal tract, suggesting that a much larger portion of the microbes present within the dust came from the pigs' faecal matter. This is in contrast with the culturing data, which suggested that the majority of the microbes were skin-associated.

One of the main results from the study showed that the bacterial microbial communities found in the dust were unique, with each farm having its own bacterial fingerprint, as shown in Figure 3. In contrast, the fungal data showed limited separation between groups, thereby indicating that the mycobiomes between pigs farms are more similar (White et al., 2019).

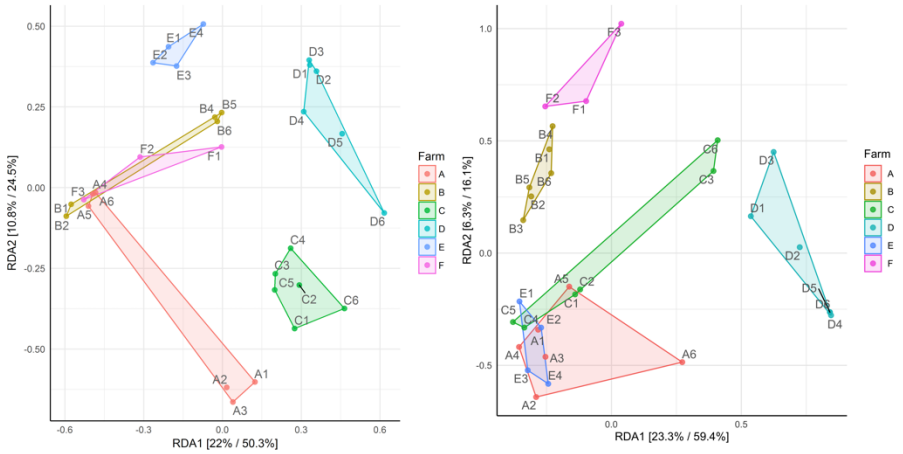

Figure 3 Constrained redundancy analysis (RDA) plots of the bacterial microbial communities present in sedimented dust observed amongst six pig farms in Denmark. The plot on the left shows the microbial community structure after sequencing. The plot on the right shows the microbial communities observed after culturing. Each dot within each plot represents the microbial communities within that sample in relation to the diversity in samples. Data taken from ***Paper 1.***

These differences in bacterial communities between different pig farms suggest that broad recommendations for reducing or modulating the bacterial communities present in pig farm dust cannot be made, due to the presence of different bacterial species between farms, and therefore a solution which could reduce a specific set of pathogens within one farm is not guaranteed to work in another. In contrast, the fungal microbial communities were found to be more closely related, and therefore methods which reduce workers' exposure to dust-borne fungi on one farm might be not applicable amongst other pig farms.

1.3. SAMPLING AIRBORNE MICROORGANISMS

Before sampling can even begin, researchers must first consider *how* they plan on collecting bioaerosols. When making a risk assessment, they have to know how they are going to characterise workers' exposure, as each sampling method has its own benefits and drawbacks, with some samplers being unsuited for studying specific risk

assessment tasks. For airborne microorganisms, a series of different sampling methodologies is available, with a series of different parameters which can be adjusted or taken into account.

Within occupational exposure studies, two main groups of air samplers are used: personal and stationary, both of which have been used during research for this Ph.D. Personal samplers are commonly used for assessing a worker's exposure to airborne microbes and microbial fragments, but face limitations regarding their limited sampling volume, and limited ability regarding tracking source exposure. In contrast, stationary samplers may be designed to sample both large and small volumes of air at higher/lower air flows, but run the risk of not being fully representative of a workers exposure, as workers typically do not work standing still in a single location. However, these devices are typically good for the analysis of source exposure, and can give further information regarding the deposition of microbial species in specific aerodynamic size bins. In addition, these samplers can collect large amounts of biomass, which can make certain downstream analyses, such as sequencing, much easier.

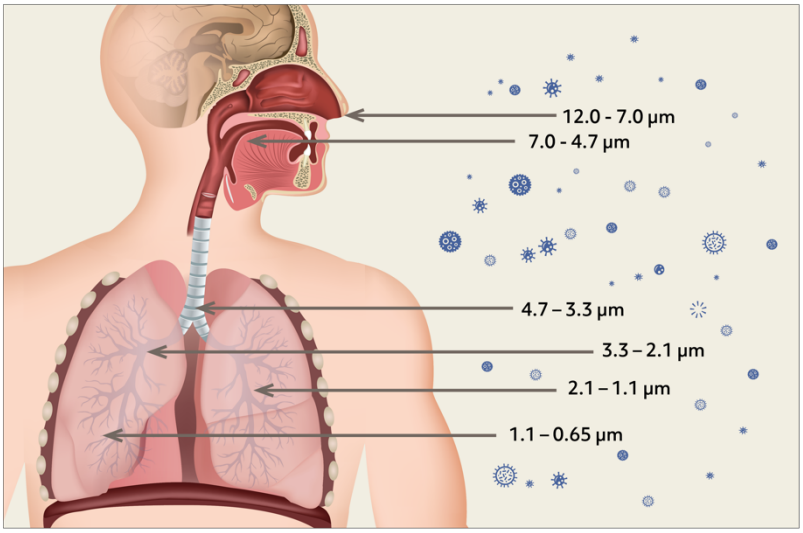

Figure 4 Illustration showing the potential deposition of airborne bacteria and fungi, and the potential deposition in the airways based on their aerodynamic diameter. Particles with a larger aerodynamic diameter will deposit in the upper airways while smaller particles can deposit in the lower lung. Figure provided by Trine Larsen.

Both personal and stationary samplers use a variety of different sampling methodologies involving active and passive sampling, and sampling matrixes. A brief discussion on the pros and cons of the different sampling methods are discussed below.

1.3.1. ACTIVE VERSUS PASSIVE SAMPLERS

During active air sampling, air is pulled into the sampler using a pump, and airborne particles are filtered onto, embedded in, or deposited on or in the sampling medium, such as liquid, agar, or a filter. In addition, the speed at which air is pulled through the sampler can be adjusted, allowing for the collection of a large amount of biomass in a relatively short amount of time, or allowing for the sampler to mimic the amount of air a person would breathe in a given period of time. However, there are limitations to using active samplers, particularly in regards to maintaining cell viability during sampling, which is discussed in greater detail further below. In particular, a high air flow during active sampling will begin to cause desiccation of the sampled material (Xu et al., 2013). In addition, as active samplers are reliant on pumps to pull air, these samplers are not always practical in certain studies, such as during cold weather conditions, where the batteries in the samplers can begin to lose power. Additionally, as the pump pulls air, the filter to which airborne particulate matter is deposited onto will eventually become clogged as the filter becomes saturated; thus the air flow through the filter will not be properly maintained.

In contrast, passive samplers rely on inertial or static electrical forces for the collection of airborne or sedimented materials (Grinshpun et al., 2015). These include samplers such as the electrostatic dust cloth (EDC), swabs, dust fall collector (DFC), and open petri dishes with agar media. The advantage of using passive samplers is that exposure can be assessed over a longer period of time, for up a month, and no pumps or other materials are required. In particular, EDCs have been used in a variety of different occupational exposure studies, including pig farms, as demonstrated in **Paper 1** (White et al., 2019), bakeries (Caetano et al., 2017), and from waste collectors (Madsen et al., 2016). For passive samplers, such as EDC cloths, the gradual accumulation of biomass or other chemical particles will eventually cause the sampler to become saturated, and at a certain point, no more material will adhere to the cloth. In addition, if the sampled bacteria and fungi are not extracted from the EDC cloth within an appropriate timeframe, then there is the risk of a loss of cell viability or culturability, which can affect downstream analyses.

In addition, there are differences between active and passive samplers in regards to their effect on cell viability. If a sampler has a high air flow, a greater amount of biomass will be accumulated, but at the same time, the sheer forces imposed upon the sampled material might damage or even kill the airborne microbes. If samples collected using these active air flows are used for culturing, biases might occur where only cells with a thicker cell wall, such as spores, will remain viable and culturable (Marthi et al., 1990). If the samples are used for sequencing, cells which are more friable, may have been overly degraded during sampling.

In contrast, for passive samplers, while there are no shear forces which might damage the cells, there is still the risk regarding dehydration, especially if the sampler is left

for a long period of time. In this case, the bacteria or fungal species which might have grown under laboratory culturing conditions, are only those which could survive extended periods of dehydration, and thus the species identified might not be fully representative of a workers exposure (Tang, 2009).

1.4. WHEN TO CHOOSE ONE SAMPLER OVER ANOTHER

1.4.1. PERSONAL OR STATIONARY SAMPLES?

An important consideration to be made when performing occupational studies is whether the samples are "personal" or "stationary" measurements. Personal samples are, as expected, specific to the person to whom the sampler is attached. These samplers tend to be small and sample the respirable fraction of air where the sampler is clipped onto the wearer. An example of a commonly utilised personal samplers includes *Gesamtstaubprobenahme* (GSP) samplers, which have been used during this Ph.D. Personal samplers tend to have a low airflow rate (<5 L/min), where the airflow can mimic the inhalation rate of the person wearing the sampler (Wang et al., 2015). The advantage of these samplers is that a worker's specific exposure can be assessed and compared with other workers, who may have other work tasks. Thus, if one worker is found to be exposed to elevated concentrations of pathogenic microorganisms, then safety recommendations regarding that specific work task can be made. However, workers may perform multiple work tasks, such as waste collectors, who both drive the truck and load the trash bins to the collection loading mechanism and thus their exposure to microorganisms during specific work tasks cannot be ascertained (Madsen et al., 2020).

Other samplers, such as stationary samplers, are placed in predetermined locations. Depending on the study design, this can be in the centre of a walkway (**Paper 3:** White et al., 2020a), or near a certain device/location (Uhrbrand et al., 2017). Stationary samplers can also be used to assess temporal variation in bioaerosol concentrations and composition (Dybwad et al., 2014). As many stationary samplers are not designed to be carried or held by a volunteer, they are not restricted by weight or size. Consequently, these samplers can be built with a much higher airflow rate, allowing for sampling of a much larger volume of air much quicker. However, stationary samplers still have limitations, which become particularly evident when sampling into a liquid. Sampling using liquid is preferred by some researchers, as it bypasses the extraction step for removing collected microorganisms from a filter or membrane. However, long sampling using a liquid has also shown that entire genera can be lost due to re-aerosolisation of the bacteria from the liquid to the air (Lemieux et al., 2019).

1.5. POST SAMPLING

1.5.1. OBTAINING SPECIES LEVEL IDENTIFICATION

Obtaining the samples is just the first hurdle; researchers have to then figure out what is in their samples. In current studies attempting to characterise microbial exposure, species and genus level identification are routinely used, with the depth of taxonomic resolution depending on the methodology. For obtaining species-level identification, some studies use species-specific chromatogenic agar (Rosen et al., 2018), proteomic spectra (**Paper 1**, White et al., 2019), or molecular tests, such as PCR (Van Cleef et al., 2015).

Like any technique, each method to obtain species-level identification has strengths and weaknesses. Chromatogenic agars, while allowing for the identification of specific species and/or antibiotic resistance by colour, are highly specific to only a few species. Molecular methods, such as PCR, can also give information about a specific species, but without modifications it does not give information regarding viability of the original sample. While an unknown sample can be processed through different multiplex PCR mixtures and/or plated on multiple chromatogenic agar types, assumptions regarding the potential presence of specific species are still required prior to running the experiments.

This runs the risk that if the sample material contains species which were not targeted, then no information about them would be obtained. This can present an issue, as rare pathogens may be present and would have been ignored when focusing on one or two more common species. This was seen in the studies by Feld et al. (2018) and **Paper 1**. In the two studies, samples from the same farms were assessed. In the study by Feld et al. (2018), the researchers focused on MRSA and used chromogenic agar to identify high concentrations of MRSA. In contrast, **Paper 1** focused on overall microbial diversity, bacterial and fungal, and found 28 pathogenic species, in concentrations far exceeding the MRSA concentrations found by Feld et al. These two studies are important, because the two combined show the strengths and weaknesses of each study, as the study by Feld et al. found the specific pathogen MRSA, whereas in **Paper 1**, several other pathogens in higher concentrations were identified.

The question regarding whether studying a specific species or conducting a broad study presents itself as a parameter which should be taken into consideration during sampling design. Would it be better to obtain very specific information, such as the presence of antibiotic resistant strains of microorganisms, which might be in low concentrations? Or would it be better to do a broad study, where you might not be able to detect low abundant, clinically relevant strains, but obtain other knowledge on species which were not considered in the original sampling design?

1.5.2. INFLAMMATORY POTENTIAL

While quantitative and qualitative data on microbial data has been obtained, this does not represent the total characterisation of microbial exposure. Other parameters may be considered beyond species-level identification. One aspect is the inflammatory potential of an offending aerosol particle after it encounters the human immune system.

As part of the innate immune system, during the exposure of airborne bacteria and fungi to the airways, immune cells will induce inflammation as a response mechanism to offending particles. During this time, immune cells will begin to secrete a variety of different cytokines and reactive oxygen species (ROS) to promote the migration of other immune cells to the area. Depending on the offending species or particle reactivity, more or less inflammation will occur. In several studies, this first-line inflammatory response has been measured using a granulocyte-like assay, where human promyelocytic leukaemia cells (HL-60) are induced to differentiate into mature granulocyte morphology. These cells are sensitive towards pathogen associated molecular patterns (PAMPs), which are the same components that the innate immune system recognises during primary infection. Upon recognition of these PAMPs, the differentiated granulocyte-like cells produce ROS. In the granulocyte assay, the production of ROS can be measured via a chemiluminescent reaction. The granulocyte assay has been used to study the inflammatory potential of a variety of different particles, including those from homes (Frankel et al., 2012), biofuel plants (Timm et al., 2009), fungi from nursing homes (Lu et al., 2020), and materials from mouldy buildings (Knudsen et al., 2017).

In **Paper 2**, the inflammatory potential of airborne *S. aureus* with and without airborne pig farm stable dust was assessed. From the results of that study, it was observed that aerosols containing pure *S. aureus* had little to no inflammation within the assay. Aerosols containing pure dust were noted to be far more inflammogenic than aerosols containing *S. aureus.* The results therefore pointed to a potential synergistic effect of dust and *S. aureus* in their inflammatory potential, as aerosols containing *S. aureus* and dust in combination were found to have a far greater inflammatory potential than either group alone.

However, these results regarding the inflammatory potential of *S. aureus* in dust should be considered conservatively. Research on ROS production *in vitro* has shown that the production of ROS during exposure to fungi is dose, species, and isolate dependent (Lu et al., 2020). Therefore, there is the possibility that other strains of *S. aureus* and other types of dust, which itself might contain other species of viable or non-viable microorganisms, will induce a different level of ROS production.

1.5.3. OTHER PARAMETERS CONSIDERED IN THIS PH.D.

Although there are many parameters occupational hygienists can study, including workers' exposure to specific species of airborne microorganisms or the inflammatory potential of these aerosols, there still remain a few areas of study for further consideration. These include biofilm formation, microbial viability, and antimycobial resistance testing. These three parameters have been the main focus of this Ph.D. thesis, and thus each of these three parameters will be discussed in greater detail during Chapters 3, 4, and 5 respectively.

1.6. ANALYSIS OF COMPLEX EXPOSURE DATA

Finally, when the data has been accumulated, the species and genera have been identified, risk parameters have been assessed. Now all that is left is to analyse the data.

Unfortunately, analysing microbial data, is one of the most difficult aspects of occupational hygiene studies, as potentially enormous datasets are generated. This has been often seen after the development of robust and high-throughput methodologies for the identification of microorganisms, and the sheer scope of data collected and generated requires the use of appropriate statistical modelling to properly analyse and visualise trends in data.

From the taxonomic information, researchers can begin to analyse the relative risk(s) pig farm workers may face based on the sampled material. Changes in the exposure, whether it be greater or fewer numbers of reads/CFUs, exposures to certain species or presence of antibiotic resistance, can be assessed and risk evaluated. The microbial diversity within a sample can be expressed as richness (number of species per sample), or other diversity indices such as Chao1, Shannon-Weaver, or Simpson, which can provide information regarding the diversity and evenness of a sample (Kim et al., 2017).

However, the analysis of exposure data can go further. In occupational exposure studies relying on amplicon sequencing or culturomics, multivariate statistically based methods and diversity indices are used to further characterise the exposure using ecological data parameters (also referred to as metadata variables). These parameters, whether they are qualitative (sampling location, weather conditions, sample type, etc.), or quantitative (temperature, number of animals per pen, sampling time, etc.), can be used in conjunction with the microbial identification data to ascertain whether correlations between the microbial exposure profile and metadata parameters exist.

From these correlations and metadata parameters, descriptive statistical techniques such as hierarchical clustering and ordination can be used to compare groups (defined

by metadata variables) of microbial exposure data. In these visualisations, the distances between samples can be based on Euclidian distance or dissimilarity (Bray-Curtis, Jaccard, etc.), where samples which are more dissimilar are clustered further apart from each other. Statistical analyses such as principal component analysis (PCA), constrained redundancy analysis (RDA), non-metric multidimensional scaling (NMDS), as well as several others, "simplify" this complex multidimensional data down to a 2D or 3D representation (Hugerth and Andersson, 2017). This has been used in several occupational studies, from comparing the nasal microbial diversity between farmers (Kraemer et al., 2018), pig farmers' homes (Vestergaard et al., 2018), and settled dust (**Paper 1**, White et al., 2019).

CHAPTER 2. BIOFILM FORMATION AS A RISK

2.1. WHAT ARE BIOFILMS?

Biofilms, defined as microbial communities which have permanently attached themselves to an abiotic or biotic-surface, are ubiquitous in nature. They occur in our mouths in the form of dental plaques, in rivers as the slime on slippery rocks, and occasionally on catheters in patients (Hall-Stoodley et al., 2004). The biofilm is, usually, composed of a variety of different bacterial and fungal species embedded within a matrix which is produced by the microbes within it (Boisvert et al., 2016). A cartoon example of a mixed microbial biofilm is shown in Figure 5. The biofilm matrix, composed of extracellular polymeric substances (EPS) containing proteins, polysaccharides, lipids, and DNA (Limoli et al., 2015), helps to protect the microbial community from external stressors, such as UV radiation (de Carvalho, 2017), desiccation (Marks et al., 2014), phagocytosis (Domenech et al., 2013), and antibiotics (Høiby et al., 2010).

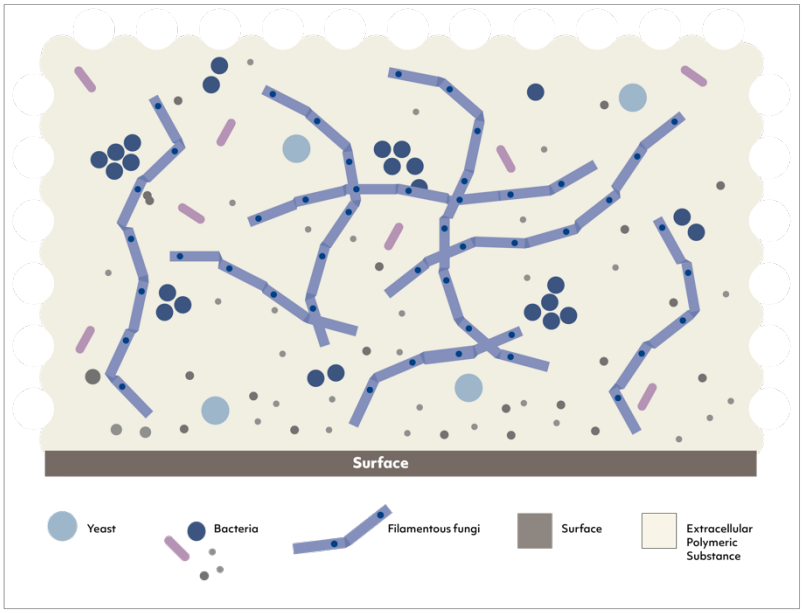

Figure 5 Schematic overview of a mixed species biofilm composed of different bacterial and fungal species embedded within a matrix composed of extracellular polymeric substance (EPS).

Due to the protective ability of the matrix, biofilms can form on medical equipment, such as catheters, dentures, and intratracheal tubing (Boisvert et al., 2016). Once the biofilm has formed, it requires treatment for removal, often requiring debridement for proper removal (Attinger and Wolcott, 2012).

2.2. FOR WHOM ARE BIOFILMS A PROBLEM?

For people with underlying health conditions, such as chronic obstructive pulmonary disorder (COPD), biofilms can form much easier in the airways, due to the hosts' reduced lung-function. Once a biofilm forms, it is very difficult for the body to naturally clear it, due to the protective layer of EPS, which prevents immune cells from clearing the contaminants. In addition, biofilms inherently have an increased resistance to antibiotics (Høiby et al., 2010).

Some species of bacteria and fungi are well-known to be biofilm formers. These include *Pseudomonas aeruginosa, S. aureus, Candida* spp. and *Aspergillus fumigatus* (Boisvert et al., 2016). Within the pig farm environment, *S. aureus* is one of the most commonly detected bacteria which is known to form biofilm (Roque et al., 2016). The presence of high concentrations of airborne *S. aureus* is worrying, as biofilms containing *S. aureus* have been found in patients with chronic lung disease (Kiedrowski et al., 2018).

2.3. BIOFILMS IN OCCUPATIONAL SETTINGS

For occupational hygiene studies, the formation of biofilms has not been a focus. This is likely due to the low rate of infections involving the formation of biofilm in workers who are exposed to biofilm formers, which itself is likely associated with the "healthy worker effect" as most manual labourers, such as pig farmers, are generally in good health. It is also possible, that medical personnel do not associate the aetiological agents for infections with the patients' occupational settings. However, more research has begun to focus on the formation of biofilms in other occupational environments or equipment, such as in face masks (Majchrzycka et al., 2018, 2017), or in highly specific environments, such as in metal working fluids (Trafny, 2013).

In regards to pig farms, biofilms have been studied, not in regards to workers' exposure to biofilm formers, but rather for their potential to act as air filters. Biofilms have been proposed to be used as a type of air filtration, where bio-filters incorporate active biofilms, in order to remove airborne bio-contaminants (Liu et al., 2020; Vyskocil et al., 2019). Circulating air is blown directly onto the surface of the biofilm and bioaerosols and other contaminants, such as ammonia, are captured and metabolised by the biofilm. While some results from these studies suggest that these filters can reduce the number of bacteria in the outlet air, others suggest that the biofilters themselves release bacteria into the outlet air (Aarnink et al., 2011; Vyskocil et al., 2019).

However, the potential for aerosolised bacteria and fungi to form biofilms is slowly beginning to be considered. A study on waste collection workers' exposure to bacteria and fungi found that workers were exposed to certain species of fungi, including *A. fumigatus* and that some of these fungal isolates displayed the ability to form biofilm (Madsen et al., 2020). This is worrying, as the potential to form biofilm of these fungal species is related to the development of potentially lethal invasive diseases, such as aspergillosis (Kaur and Singh, 2014).

2.3.1. BIOFILMS IN PIG FARMS

Unfortunately, knowledge regarding the potential for airborne microorganisms within pig farms to form biofilms is lacking. To address this, **Paper 2** focuses on *S. aureus,* a pathogenic bacterial species commonly encountered within pig farms (Feld et al., 2018; Guardabassi et al., 2007), and assesses its ability to form biofilms after aerosolisation in or without the presence of sterile airborne farm dust. *S. aureus* was chosen as the organism of study, not only because it is commonly encountered in the pig farm environment, but also because it is encountered in non-farm environments, such as offices and homes as both antibiotic-sensitive and -resistant strains (Fritz et al., 2014; Madsen et al., 2018b).

Results from the study performed in **Paper 2** showed, that when *S. aureus* was aerosolised simultaneously with sterile farm dust, more biofilm formed than in aerosolised samples of *S. aureus* without the presence of sterile farm dust. Thus presenting evidence that the presence of dust has a positive influence on *S. aureus* forming biofilms after aerosolisation (White et al., 2020b). The results from **Paper 2** highlight that removal of dust, or reducing dust generation, are aspects which can help to reduce the formation of biofilms within the pig farm environment. However, it should be noted that *S. aureus* formed less biofilm when subjected to aerosolisation in comparison with *S. aureus* which was stored in liquid prior to the biofilm assay.

Additionally, while not part of the scope of **Paper 2**, it was found that the biofilms formed by *S. aureus* after aerosolisation presented markedly different morphology compared with biofilms formed by *S. aureus* originally grown as planktonic cultures before biofilm testing, as shown in Figure 6.

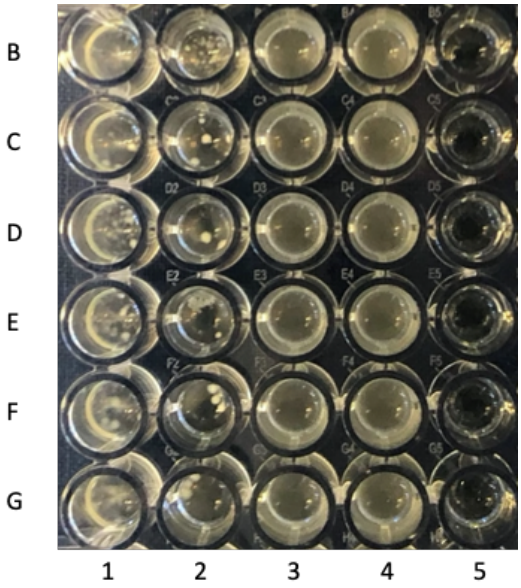

Figure 6 Biofilms produced by Staphylococcus aureus *which was aerosolised with dust (columns 1 and 2) grown in tryptic soya broth (TSB). Column 3 shows biofilms produced by* S. aureus *which were taken from the bubble generator. Columns 4 and 5 represent the positive control (*S. aureus *from a planktonic culture) and negative control (TSB media without inoculation), respectively. Note the lumpy, uneven morphology of the biofilms produced by* S. aureus *after aerosolisation seen in columns 1 and 2 in contrast to the smooth biofilms seen in columns 3 and 4.*

As changes in biofilm morphology were not part of the scope of **Paper 2**, the reasons as to why the biofilms were so markedly different are still unclear. However, these differences may be attributed to changes in gene expression post-aerosolisation, as other studies which have investigated aerosolised bacteria found changes in gene expression involved in a variety of different metabolic pathways (Ng et al., 2018). A recent study looking at the formation of biofilm by *Streptococcus pneumoniae* found a significant difference in the morphology of their biofilms post exposure to Asian sand dust particles (Yadav et al., 2020). This suggests that the presence of dust could have also influenced the changes in biofilm architecture.

In addition, although the study performed in **Paper 2** found that the presence of airborne farm dust influenced the production of biofilm, these results are still limited; in real life situations, there will not only be a single species of bacteria in bioaerosols. Pig farms aerosols are complex, composed of both bacterial and fungal species, which are both dead and alive (Roque et al., 2016; **Paper 2**, White et al., 2020a). Therefore, the results from this study are limited, as the presence of only a single airborne species was assessed, along with only a single type of dust. Other *in vitro* studies which have assessed preferential aerosolisation of bacteria have shown that some bacteria, such

as *Pseudomonas aeruginosa*, are preferentially aerosolised (Perrott et al., 2017). If certain species of bacteria and fungi are preferentially aerosolised, it would be interesting to determine which species in complex environmental samples would preferentially form biofilm after settling.

CHAPTER 3. ANTIMYCOBIAL RESISTANCE AS A RISK FACTOR

After Dr. Alexander Fleming accidentally discovered the antibiotic penicillin in 1928 (Tan and Tatsumura, 2015), we were finally able to treat bacterial infections, which would otherwise have proven fatal. However, the good days were not to last, as the moment humans started using antimicrobials, an anthropogenically induced evolutionary arms race between microbes and antimicrobials began, with resistance to antibiotics appearing in clinical samples after only a few years after their discovery (Ventola, 2015).

The emergence and increased prevalence of antimicrobial resistance (AMR) to commonly used antimicrobials is a growing problem around the world, as the overuse of antimicrobials in agricultural and medical settings has been linked to the development of AMR in both target and non-target microorganisms (Snelders et al., 2009). This is of great concern, as there has been a worrying trend of increasing numbers of environmental and clinical bacteria and fungi exhibiting resistance while fewer and fewer antibiotics are introduced to the market each year (Ventola, 2015).

3.1. RESISTANCE TO ANTIBACTERIALS IN PIG FARMS

One of the most common AMR species of bacteria in the pig farm environment is livestock-associated methicillin resistant *Staphylococcus aureus* (LA-MRSA), which has been found in e.g. Denmark (DANMAP, 2011), Germany (Goerge et al., 2017), the Netherlands (Van Cleef et al., 2015), and Belgium (Garcia-Graells et al., 2012). Although the number of infections caused by LA-MRSA is quite low in Denmark (DANMAP, 2019), it is still considered an occupational health risk, as a study by Angen et al. (2017) found that MRSA negative volunteers became positive after performing work tasks in a LA-MRSA positive pig farm. From that study, the authors concluded that LA-MRSA is airborne within pig farms, which allows it to easily be spread and disseminated further in the environment. In addition, the incidence rate amongst workers in pig farms, both farmers and veterinarians, is high, with some studies reporting up to 57.9% of workers testing positive for LA-MRSA (Reynaga et al., 2016).

Another class of AMR bacteria found in pig farms are the vancomycin-resistant Enterococci (VRE) (Nilsson, 2012; Seo et al., 2005). However, although enterococci from pig farms test positive as being VRE, there does not appear to be a high risk of having nasal colonisation, as a study on spray irrigation workers found that neither the spray workers nor the office worker control group had VRE nasal colonisation (Rosenberg Goldstein et al., 2014).

3.2. RESISTANCE TO ANTIMYCOBIALS IN PIG FARMS

Of course, AMR is not restricted to just bacteria; several species of fungi are also reported to exhibit AMR. This is commonly attributed to the (over)-use of antimycobial agents in agricultural settings, which are primarily used to prevent fungal infections in crops (Snelders et al., 2009). This emergence of AMR in non-target fungi is of particular concern as some human fungal pathogens, including *Aspergillus fumigatus*, *A. niger,* and *A. terreus,* have also developed resistance to agricultural antimycobials (Mortensen et al., 2010). This is a significant issue, as the resistance they gained from the agricultural antimycobials has conferred cross resistance to medical antimycobials (Azevedo et al., 2015). AMR resistance in environmental fungal isolates, such as members of the genus *Aspergillus,* is of great concern, as although fungal infections are rare, the mortality rate of invasive aspergillosis ranges from 30-90% (Brown et al., 2012).

However, the amount of research regarding antimycobial-resistant fungi in occupational settings is extremely scarce. In a study by Caetano et al. (2017), sedimented dust from Portuguese bakeries was found to contain high concentrations of multiple genera of fungi, including *Cladosporium*, *Penicillium,* and *Chrysosporium* which displayed resistance to triazoles (Caetano et al., 2017). From the published literature, it appears that testing for AMR fungi has not been a focus for occupational risk studies. To address this lack of knowledge, the antimycobial resistance in environmental fungal isolates from a pig farm was explored (**Paper 4**). These fungi were isolated from faecal matter, sedimented dust, airborne particles, and feed material. Airborne fungal particles and sedimented dust were collected as these sample types represent a workers potential exposure in occupational settings. The feed material was chosen as triazoles, which have been overused in the production of grain and grass, and thus fungi from these grains have experienced a selection pressure for azole resistance (Azevedo et al., 2015). From the data gather during this thesis (**Paper 1, Paper 3**), it was found that a large number of the airborne fungi were associated with the gastrointestinal tract of pigs. Therefore, the faecal matter presented a potential source of fungi, which might have resistance to antimycobials, as the pigs' natural fungal gastrointestinal flora could have been exposed to agricultural antimycobials and/or AMR fungi via the feed.

During the studied performed in **Paper 4**, over 350 isolates of fungi were found, covering six genera, but only fungal isolates from the genus *Aspergillus* were tested for their potential resistance to three classes of antimycobials commonly used to treat patients. The classes of antimycobials included two types of triazoles (itraconazole and voriconazole), a polyene (amphotericin B), and an echinocandin (caspofungin acetate). Fungal isolates were tested for the resistance to the aforementioned antimycobials in accordance with the European Committee for Antimicrobial Susceptibility Testing (EUCAST) guidelines.

Worryingly, 27.9% of the tested isolates were resistant to at least one antimycobial and 11.6% were resistant to multiple classes of antimycobials. An example of the antimicrobial resistance profiles to Amphotericin B is shown in Figure 7. While none of the *A. fumigatus* isolates were resistant to the tested antimycobials, *A. niger, A. terreus,* and *A. versicolor* exhibited resistance towards all four tested antimycobials. Although *A. fumigatus* is the most common causative organism for aspergillosis, the other species mentioned can also cause invasive disease amongst susceptible populations (Hachem et al., 2014; Person et al., 2010; Tashiro et al., 2011).

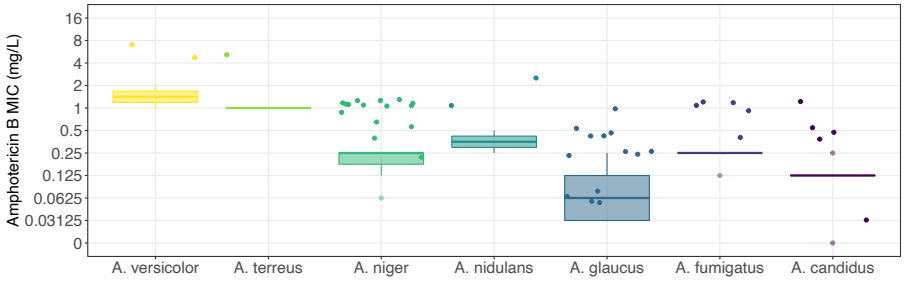

Figure 7 Antimycobial resistance profiles of seven species of Aspergillus *towards Amphotericin B tested using the European Committee for Antimicrobial Susceptibility Testing (EUCAST) protocol. Each dot represents an isolate's minimum inhibitory concentration (MIC). Isolates with a MIC above 2 are generally considered to be resistant. This figure is taken from* ***Paper 4****.*

The presence of such as high percentage of antimycobial-resistant fungi which can cause such invasive diseases is worrying, especially given that these results are only from the species within the genus *Aspergillus.*

Unexpectedly, during **Paper 4**, some fungal isolates also exhibited a phenomenon known as paradoxical resistance. In a "normal" broth microdilution assay, antibiotic sensitive strains will stop growing once the minimum inhibitory concentration (MIC) is reached, and antibiotic resistant strains will continue to grow at higher concentrations of antibiotic. In strains which exhibit paradoxical resistance, the fungi will reach the MIC and stop growing. However, as the concentration of antibiotic in the assay increases, the fungi begin to paradoxically begin growing again. A schematic overview of this is shown in Figure 8.

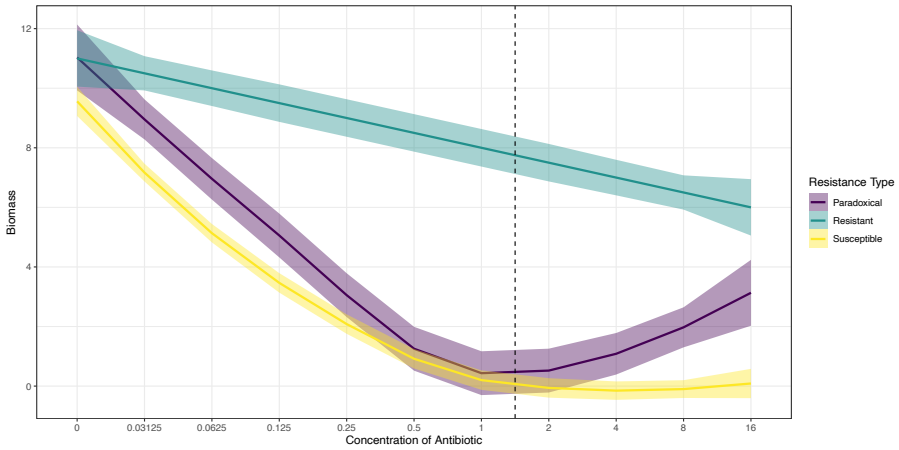

Figure 8 Example overview of the different resistance categories, where increasing concentrations of antibiotics after the "normal" minimum inhibitory concentration (shown in the graph as the vertical dotted line) do not inhibit microbial growth (purple line). This is in contrast to the resistant (aqua) and susceptible (yellow) phenotypes for antimicrobial resistance.

The reasons for this trend could be due to a variety of different molecular causes, with mechanisms such as the increased production of cell wall chitin (Stevens et al., 2006). However, at the time of writing, no established potential mechanism for this phenomenon has been proposed for paradoxical resistance to triazoles within the genus *Aspergillus.*

The overall lack of knowledge on antimycobial resistance within pig farms is a problem, as pig farms are known to contain a variety of different pathogenic fungal species, which have the potential to deposit in all regions of the airways (White et al., 2020a). While fungi from the genus *Aspergillus* were tested for their antimycobial resistance during **Paper 4**, there were other species of pathogenic fungi, including *Lichtheimia corymbifera* and *Rhizomucor pusillus,* which were not tested for their resistance, even though they are known to cause disease in humans (Skiada et al., 2011). Therefore, it is of paramount importance, considering the spread of antimycobial resistance in agricultural settings that pig farm workers' exposure to antimycobial-resistant fungi should be considered during occupational exposure studies.

CHAPTER 4. VIABILITY AS A RISK FACTOR

Considering viability of microbes in occupational exposure studies is not a novel concept. Older studies used techniques such as staining with fluorescent chemicals or probes to assess via microscopy the number of damaged and fragmented bacterial and fungal cells within a sample (Chi and Li, 2005; Heldal et al., 1996). However, these techniques have slowly lost favour over the last decade, with only a few studies considering viability in their study design (Lawniczek-Walczyk et al., 2013; White et al., 2020a). In several studies the assessment of workers' exposure to pathogens or non-pathogenic species is performed using culturing (Lu et al., 2020; Viegas et al., 2013), or by molecular based methods (Degois et al., 2017).

For an active infection to occur in a host, the pathogen(s), bacterial or fungal or both, have to be able to replicate and produce copies of themselves, thereby prolonging the infection. However, diseases caused by microorganisms are not necessarily always infections. Some fungi, such as *Alternaria alternata,* are able to cause an allergic reaction in susceptible hosts, even if the fungi itself is non-viable (Mitakakis et al., 2003). In contrast, the viable spores of other fungal species, such as *Aspergillus fumigatus,* will produce a higher immune response in murine models compared with heat-killed spores (Croston et al., 2016). Therefore, it is important to consider whether the airborne microorganisms are viable or dead, as the immune response that workers may experience may depend not only on the species, but in regards to that species' viability as well.

4.1. CULTURABLE OR VIABLE?

There is an important distinction between viability and culturability. Culturability is defined as whether something can or will grow under laboratory conditions, whereas viability is defined as the cell itself being able to perform metabolic activities with the ability to replicate. This is in distinction to non-viable cells which are damaged and unable to perform metabolic functions and are unable to replicate (Barer and Harwood, 1999). However, to further complicate things, there is a third category which lies between the viable and culturable states.

In the 1980s, researchers discovered that several bacterial species have evolved to enter a state of dormancy, called the Viable, But Not-Culturable (VBNC) state (Xu et al., 1982). VBNC cells are not metabolically active, but possess the ability to "resuscitate" at a later point and become metabolically active and begin to replicate once again (Figure 9). Examples of bacterial species which are known to enter the

VBNC state include *Salmonella enterica* (Gupte et al., 2003), *Staphylococcus aureus* (Bai et al., 2019) and *Vibrio vulnificus* (Nowakowska and Oliver, 2013).

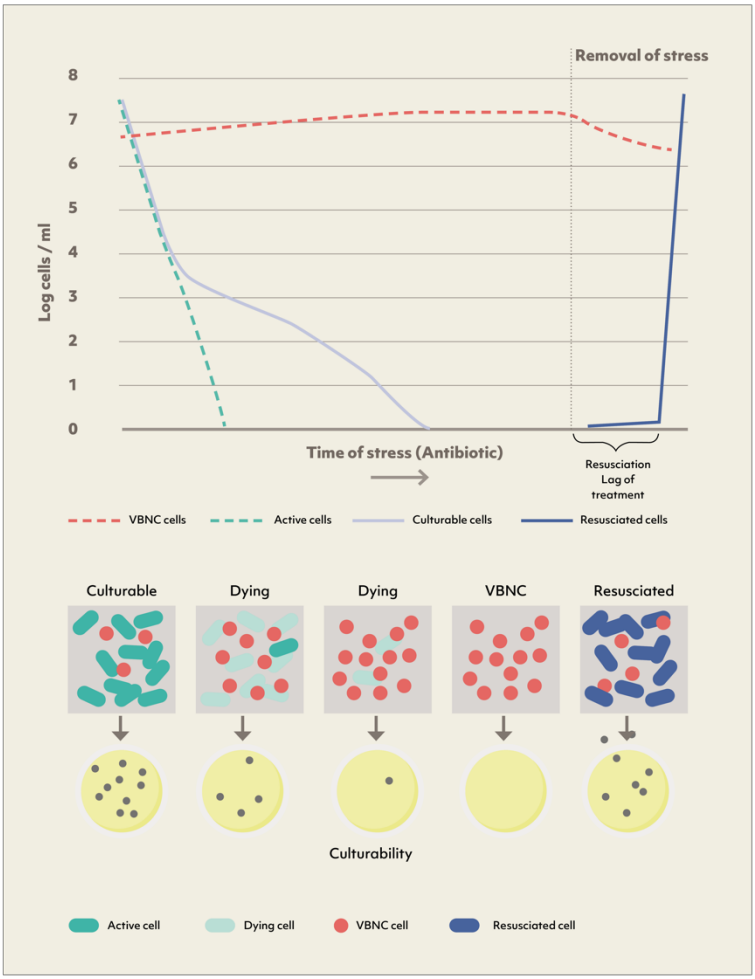

Figure 9 Schematic overview of a population of cells which are being killed off by a stressor (in this case an antibiotic). Within the total population, there exists a subpopulation of cells which are viable, but not culturable (VBNC). After removal of the stressor (dotted grey line), some of the VBNC cells begin to resuscitate and will regain their ability to be cultured on media. Figured adapted from (Ayrapetyan et al., 2018)*. Figure provided by Trine Larsen.*

In the present Ph.D. the viability of bioaerosols was considered in two articles. In **Paper 2**, *in vitro* aerosols of *Staphylococcus aureus* were generated and assessed for their viability during aerosolisation with and without the presence of airborne dust by using a modified qPCR. In **Paper 3**, complex fungal aerosols were collected from a

pig farm and the viability of these fungal aerosols was considered in relation to their taxonomy and their potential deposition in workers' airways. These two topics, viability of *S. aureus* and complex fungal aerosols are discussed in further detail in the subsections 4.2 and 4.3, respectively.

To begin, it should be noted that studying the VBNC state of aerosols is difficult. One of the issues is that aerosolisation and sampling of bioaerosols bestow stresses and small injuries upon the cells, which in turn decreases the viability of cells (Heidelberg et al., 1997; Thomas et al., 2011). However, small modifications to commonly used methods for studying bacterial contamination are available and have allowed for the study of VBNC bioaerosols. One such method, which has been used in this Ph.D., utilises viability qPCR, where prior to DNA extraction, free DNA is chemically modified by using DNA inter-chelating agents, such as propidium monoazide (PMA) or ethidium monoazide (EMA). These chemicals bind to free DNA or DNA in membrane damaged cells, and upon exposure to light, inter-chelate with the DNA. The resulting PMA/EMA-modified DNA is unable to be amplified during PCR, allowing for researchers to make differential analyses regarding treated and non-treated samples (Emerson et al., 2017).

4.2. VIABILITY AND POTENTIAL DEPOSITION IN THE AIRWAYS

Every time we take a breath, we inhale particulate matter, whether it be our own skin cells, soot, dust, or airborne microorganisms, such as bacteria and fungi. For most people, the inhalation of airborne dust and other particulate matter is not of great concern, due to the removal of these foreign bodies by the immune system (Schlesinger, 1988).

However, although these compounds are being inhaled, they will not all deposit uniformly within the lungs. This is due to the particles' aerodynamic diameter (D_g). The D_g influences not only how quickly an airborne particle will move through the air column, but will also determine the potential deposition of the particle in our airways. Particles with a large D_g (>4.7μm) will deposit in the upper respiratory tract (nasal cavity and pharynx), whereas particles with a small D_g (<1.0μm) can deposit in the alveoli (Jabbal et al., 2017).

Knowledge on where in the airways a particle may deposit is vital, especially regarding microorganisms, as the location within the airways a bacteria or fungus deposits has an impact on the potential disease(s) caused. For members of the genus *Aspergillus*, infections in the upper respiratory tract can cause allergic sinusitis while lower respiratory tract infections can cause the formation of aspergillomas even though the aetiological agent is the same (Barac et al., 2018; Shah and Panjabi, 2016). For bacteria, organisms such as *Streptococcus pyogenes* can cause pharyngitis or pneumonia depending on how deep in the airways the bacterium is able to penetrate (Dasaraju and Liu, 1996).

Although researchers know the diameters of bacterial and fungal cells or spores, this is not enough information to determine where in the airways specific species may deposit. For example, a study performed by Madsen et. al (2018) showed that airborne MRSA from a pig farm were mostly associated with larger particle sizes 7.0 – 12.0 µm (Madsen et al., 2018a). Although *S. aureus* by itself has a diameter between 0.5 and 1 µm (Foster, 1996), it is not necessarily airborne as single particles, and may be associated with other bacteria, or with larger dust particles.

In pig farms, knowledge on the potential respiratory deposition of viable and non-viable fungi is lacking. To address this gap in knowledge, during the study performed in **Paper 3**, the potential deposition of fungal genera and species in the airways of pig farm workers was assessed in regards to their culturability, viability, and inter/intra species composition over multiple sampling days (White et al., 2020a).

Air samples were taken from the same pig farm over the winter and summer of 2019, using two types of Andersen Cascade Impactors (ACI): the six-stage viable ACI (ACI-6) and the non-viable eight-stage ACI (ACI-8). The ACI-6 was used for collecting culturable airborne fungal particles on agar plates and the ACI-8 was used for collecting airborne fungal particles onto glass fibre (GF) filters. Afterwards the fungi present on the GF were compared for differences in the viable and non-viable airborne fungi.

In Figure 10, the different genera and phyla of fungi identified using the ACI-8 are shown in regards to their viability status, and in which size fractions those genera were found. The results show a tendency for most viable fungal genera to be mostly associated with larger particle sizes.

Detailed analysis on the viable and non-viable fungi from within the pig farm revealed that a large proportion (27%) of the airborne fungal genera was non-viable. Many of these non-viable fungi included genera, whose members are known to be associated with the pigs' gastrointestinal flora, such as *Kazachstania* and *Vishniacozyma* (Ramayo-Caldas et al., 2020; Urubschurov et al., 2011; **Paper 1**, White et al., 2019). Of the viable fungal genera, these included those with known allergenic potential, such as *Aureobasidium* and *Cladosporium,* and pathogens, such as *Aspergillus* and *Trichosporon* (Ausschuss für Biologische Arbeitsstoffe, 2016).

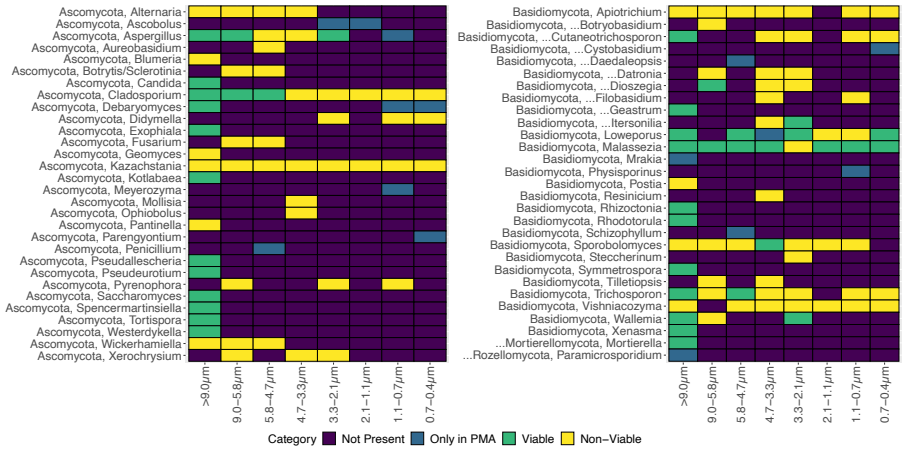

Figure 10 The genera and phyla of fungi identified after viability sequencing analysis. The colour of each box corresponds to the viability and or presence of the fungi identified within that size fraction (yellow being non-viable, green being viable, purple being not present in that sample, and blue being genera only identified in PMA treated samples). Data adapted from ***Paper 3****.*

The results from **Paper 3** suggested that workers were exposed to culturable airborne fungal particles associated with particles with a geometric aerodynamic diameter between 3.7 and 4.6µm. However, analysis of the total fungal exposure by viability sequencing revealed that the airborne fungi were mostly associated with smaller particles of around 2.1µm (White et al., 2020a). This suggests that although most of the culturable, and therefore viable, fungi would deposit in the mid-lungs, many smaller fragmented and dead fungal particles would deposit in the lower-lungs.

4.3. VIABILITY OF AIRBORNE *STAPHYLOCOCCUS AUREUS*

In **Paper 2**, the bacterial species *Staphylococcus aureus* was assessed for its culturability and viability after aerosolisation. As has been mentioned throughout this Ph.D., *S. aureus* is often found in pig farm dust, both in its antibiotic-resistant and susceptible forms (Brennan et al., 2016; Masclaux et al., 2013).

Earlier work has shown that *S. epidermidis* has a higher culturability when aerosolised with dust (Møller, 2018, unpublished). As *S. aureus* is commonly isolated from pig stable dust, I was curious as to whether the presence of dust would have a similar positive effect on the viability or culturability of *S. aureus* post-aerosolisation.

In order to first study the survival of *S. aureus* once airborne, it was first necessary to choose a method of aerosolising the cells without simultaneously damaging them. This was achieved by bubble generation, which has been suggested to be a “gentler” method of aerosolisation in contrast with other methods, such as nebulisation, which

can damage the cell wall of bacteria, resulting in a loss of viability (Mainelis et al., 2005; Reponen et al., 1997).

The results from this study showed that the culturability of *S. aureus* was significantly greater when aerosolised in the presence of airborne dust, thus suggesting that the presence of dust has some sort of a protective effect. What was very interesting was that the results from **Paper 2** gave evidence towards induction of the VBNC state in *S. aureus* post-aerosolisation, as there was observed to be a significant effect on the culturability, but not on the viability.

This result is of concern, as researchers often rely on culture-based methods for considering *S. aureus* contamination in pig farms (Feld et al., 2018; Madsen et al., 2019). Therefore, if aerosolisation induces the VBNC state in *S. aureus*, then there is a strong likelihood that the "true" rate of exposure to viable *S. aureus* is much higher than previously thought.

Another reason for concern regarding the VBNC state in bioaerosols is due to their potential to cause long-term infections in hosts. VBNC cells have been referred to as persister cells due to their ability to persist within a host, even after treatment with antibiotics (Kim et al., 2018). Although the cells themselves may not contain antibiotic-resistance gene elements and are therefore categorised as susceptible to antibiotics, due to their metabolism, the antibiotics may not affect the inactive cells (Nowakowska and Oliver, 2013). This is an important issue within the medical sciences, as cells which are not killed by antibiotics during initial treatment might reactivate, and thus can cause recurring infections (Li et al., 2014).

CHAPTER 5. CONCLUDING REMARKS AND PERSPECTIVES

Limited knowledge on the airborne microbial communities from pig farms and limited exposure characterisation methods are two of the main bottlenecks for obtaining a proper risk evaluation for occupational exposure in pig farms. The main objectives of this Ph.D. were to consider further parameters when conducting occupational risk assessments for pig farmers, which included the following:

- Biofilm formation
- Antimycobial resistance
- Microbial viability

BIOFILMS: As part of **Paper 2**, the species *S. aureus* was chosen for testing its potential to form biofilm, as *S. aureus* is commonly found in dust from pig farms. In this study, I found that more biofilm was formed by *S. aureus* when aerosols were generated simultaneously with dust. These results suggest, that the reduction of dust within the pig farm environment will reduce the potential for aerosolised *S. aureus* to form biofilms once settled.

However, although the presence of dust was considered in regards to the formation of a biofilm, the study itself takes a simplistic view of bioaerosols. Depending on the environment, bioaerosols *in natura* are more complex, composed of dust particles from multiple sources, skin flakes, bacteria, fungi, viruses, etc. Therefore, it is worth considering the potential of complex aerosols to form biofilm in an assay which mimics the human airway. In particular, focus should be on determining which species were present in the aerosols before biofilm formation, and comparing those with the species which are detected actively growing and producing the biofilm afterwards. This can help give further information regarding which species might pose a greater occupational health risk, in regards to biofilm formation.

VIABILITY: As part of **Papers 2** and **3**, the viability of airborne bacteria and fungi were considered during *in vitro* and *in natura* studies, respectively. Due to the relative novelty of the method for testing viability of airborne microorganisms, there is no consensus regarding appropriate incubation time, sampling material, light exposure time, or DNA extraction methodology. Despite this, the results from this Ph.D. have suggested that the exposure workers may face to viable microbes is greater than what was previously thought, due to the potential presence of viable, but not culturable (VBNC) *S. aureus* in pig farms. In particular, occupational hygienists should consider studying whether workers are exposed to VBNC bacteria or fungi, to help determine the “true” level of exposure microbial exposure. In addition, it is known that VBNC

cells are less susceptible to antibiotic treatment compared with active cells. Therefore if workers are treated for an infection derived from an exposure to active and VBNC cells, traditional treatment therapies might not be appropriate for the eradication of the infection.

To do so, it would be of great aid to consider using DNA chelating agents in combination with molecular methods, such as sequencing or qPCR, to ascertain which species or genera are viable at the time of sampling, as this may change the way that the data are interpreted afterwards.

ANTIMYCOBIAL RESISTANCE: The results from **Paper 4** give further evidence regarding the presence, and spread, of fungi which are resistant to antimycobial drugs used in patients with disseminated fungal disease. In other occupational settings where workers are at an increased risk of being exposed to high concentrations of airborne fungal species, researchers should consider testing for resistance to antimycobial drugs, so as to aid in the surveillance of the spread of AMR fungi.

Whilst this Ph.D. focused on workers' exposure to microorganisms, there is another aspect which could have been studied, namely workers' exposure to viruses. It is well known that people in close contact with livestock increases the chance for a zoonotic event to occur (Holt et al., 2016). As the coronavirus pandemic continues, workers' potential exposure to viruses should not be ignored, especially with reports of viruses exhibiting potential for zoonotic transmission, most recently (as of writing) with the emergence of a swine influenza virus in pig herds in China (Sun et al., 2020).

Overall, while this Ph.D. was performed focusing on pig farm workers' exposure, the applicability of the methods used in this Ph.D. are not limited to just pig farms. Studies focusing on other occupational environments, such as wastewater treatment plants, greenhouses, nursing homes, and waste sorting facilities can appreciate the methods suggested in this Ph.D.

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APPENDICES

Appendix A. **Microbial Species and Biodiversity in Settling Dust Within and Between Pig Farms**

John Kerr White, Jeppe Lund Nielsen, and Anne Mette Madsen (2019)

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Appendix B. **Impact of Dust on Airborne *Staphylococcus aureus'* Viability, Culturability, Inflammogenicity, and Biofilm Forming Capacity**

John Kerr White, Jeppe Lund Nielsen, Cecilie Møller Larsen, and Anne Mette Madsen

Accepted for Publication in *International Journal of Hygiene and Environmental Health*

Appendix C. **Potential Respiratory Deposition and Species Composition of Airborne Culturable, Viable, and Non-Viable Fungi during Occupancy in a Pig Farm**

John Kerr White, Jeppe Lund Nielsen, and Anne Mette Madsen (2020)

Published in *Atmosphere*

Appendix D. **Paradoxical and Classical Antifungal Resistance in Environmental Isolates of *Aspergillus***

John Kerr White, Jeppe Lund Nielsen, Jan Struckmann Poulsen, and Anne Mette Madsen

In Preparation

ISSN (online): 2446-1636
ISBN (online): 978-87-7210-697-7

AALBORG UNIVERSITY PRESS